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Serotonin effects in the crab *Neohelice granulata*: Possible involvement of two types of receptors in peripheral tissues

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ABSTRACT

In crustaceans, serotonin (5-HT) controls various physiological processes, such as hormonal secretion, color changes, reproduction, and metabolism. Since 5-HT injections cause hyperglycemia, this study was designed to further investigate this action of 5-HT in the crab *Neohelice granulate*, fed with a high-carbohydrate (HC) or a high-protein (HP) diet. The effects of pre-treatment with mammalian 5-HT receptor antagonists, cyproheptadine and methiothepin, were also investigated. A series of *in vivo* experiments with ^{3}H-5-HT was carried out in order to investigate the presence of putative receptors in peripheral tissues. Since gills were the tissue with the highest labeling in *in vivo* experiments, *in vitro* studies with isolated anterior and posterior gills were also conducted. Cyproheptadine blocked the hyperglycemic effect of 5-HT in HP-fed crabs. Methiothepin reduced glycogen levels in the anterior gills of HP crabs and partially blocked the 5-HT-like posture. The injection of ^{3}H-5-HT identified specific binding sites in all the tissues studied and revealed that the binding can be influenced by the type of diet administered to the crabs. Incubation of the anterior and posterior gills with ^{3}H-5-HT and 5-HT confirmed the specificity of the binding sites. Both antagonists inhibited ^{3}H-5-HT binding. In conclusion, this study highlights the importance of serotonin in the control of glucose homeostasis in crustaceans and provides evidences of at least two types of 5-HT binding sites in peripheral tissues. Further studies are necessary to identify the structure of these receptors and their signaling pathways.

1. Introduction

Serotonin (5-HT, 5-hydroxytryptamine) is a ubiquitous indoleamine found in microorganisms, plants and animals (Lam and Heisler, 2007; Pytliak et al., 2011). In crustaceans, 5-HT is present in the central and peripheral nervous system, including the eyestalk and neuroendocrine organs, especially the pericardial organs (Fingerman et al., 1994; Basu and Kravitz, 2003; Liu et al., 2008). Serotonin can act as a neurotransmitter, neuromodulator, or neurohormone, controlling various neuronal and physiological processes, such as neuronal activity, neurogenesis, behavior, color changes, osmotic adjustments, and reproduction (Fingerman et al., 1994; Morris, 2001; Meeratana et al., 2006; Ongvarrasopone et al., 2006; Liu et al., 2008; Zhang et al., 2011; Wu and Cooper, 2012). Some of these actions of 5-HT are explained by the secretion of peptide hormones from neuroendocrine organs, such as molt-inhibiting hormone, gonad-stimulating hormone, or red-pigment dispensing hormone (Fingerman and Nagabhushanam, 1992; Fingerman et al., 1994; Tiu et al., 2005; Meeratana et al., 2006; Siangcham et al., 2013). Because it can stimulate reproduction and larval transformation, some studies suggested the use of 5-HT, or its precursor tryptophan, as a food or water supplement to commercial crustaceans (Tangvuthipong and Damrongphol, 2006; Chettri et al., 2007; Laranja et al., 2010). Another well-known effect is the typical postural alteration caused by the injection of 5-HT in decapods (Beltz, 1988; Vinagre et al., 2004; Bacque-Cazenave et al., 2013).

Serotonin plays an important role in the control of carbohydrate metabolism in crustaceans, since 5-HT injections cause an increase in the levels of glucose in the hemolymph (Bauchau and Mengeot, 1966; Lee et al., 2000; Komali et al., 2005; Reddy and Pushpalatha, 2007; Sathyanandam et al., 2008). Some studies report that the hyperglycemic effect of 5-HT injections is preceded by an increase in the levels of crustacean hyperglycemic hormone (CHH) in the hemolymph (Lee et al., 2001; Santos et al., 2001; Sathyanandam et al., 2008). CHH is considered the main hormone involved in the control of glucose-circulating levels in crustaceans (Fanjul-Moles, 2006; Chung et al., 2010; Webster et al., 2012). The hyperglycemic effect of CHH is associated with increased glycogenolysis in muscle tissue and the hepatopancreas, caused by inhibition of glycogen synthase and activation of glycogen phosphorylase

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(Sedlmeier, 1982, 1985; Nagai et al., 2011). In *Macrobrachium malcolmsonii*, 5-HT injection caused hyperglycemia associated with a decrease in glycogen concentration, and an increase in phosphorylase activity in the hepatopancreas of intact, but not eyestalk-ablated, prawns, suggesting that these effects are related to CHH (Komali et al., 2005).

The burrowing, semiterrestrial crab *Neohelice granulata* is a key species in South American salt marshes, mudflats, and estuaries, ranging from Lagoa de Araruana (Brazil) to San José Gulf (Argentina) (Spivak, 2010). Since the habitat of *N. granulata* is characterized by numerous environmental changes, which may occur on a seasonal or even daily basis, including alterations in temperature, photoperiodicity, partial oxygen pressure, food availability, and composition, and salinity, this crab has evolved a great capability of dealing with different types of environmental stresses (Kucharski and Da Silva, 1991b; Vinagre and Da Silva, 2002; Spivak, 2010). Therefore, *N. granulata* is considered an emergent animal model for biochemical, physiological, and ecological research in South America (Spivak, 2010). Following the pioneering work of Kucharski and Da Silva (1991a), further studies have shown that when *N. granulata* is fed with raw meat (high-protein diet) or boiled rice (carbohydrate-rich diet), the metabolic differences imposed by the diet regimen affect the way this species of crab deals with various types of stresses induced under laboratory conditions (Vinagre and Da Silva, 1992; Schein et al., 2004; Pellegrino et al., 2008; Marqueze et al., 2011). A greater understanding of the metabolic and physiological effects of different types of diets in decapods is necessary to predict the impact of the alterations in natural food resources, caused by alterations in other environmental parameters, such as warming, in wild species as well as to forecast the effects of artificial diets in commercial species. To our knowledge, the majority of the studies about different types of diets in commercial species of decapods focus mainly on zootechnical performances such as survival rate, weight gain, growth, feed conversion ratio, and protein efficiency, while there are few studies about the effects of different types of diets on metabolical and physiological parameters. In this sense, the detailed study of the metabolical alterations induced by different types of diets in *N. granulata* can contribute to further advance the knowledge of physiology of nutrition in crustaceans, furnishing nutritional strategies for aquaculture.

When fed with a carbohydrate-rich (HC) diet, *N. granulata* develops higher levels of glucose in the hemolymph and of glycogen in the hepatopancreas and muscle than when it is fed with a high-protein (HP) diet (Kucharski and Da Silva, 1991a; Vinagre and Da Silva, 1992, 2002). In *N. granulata* crabs fed with an HP or HC diet, 5-HT injections caused a dose-dependent increase in circulating glucose levels in intact as well as in eyestalkless crabs (Vinagre, 1999; Zanotelli et al., 2002). This hyperglycemic effect was also obtained with fluoxetine, a selective 5-HT reuptake inhibitor (Santos et al., 2001). In the hepatopancreas of *N. granulata*, 5-HT injections decreased free glucose levels in intact crabs fed with either HC or HP diet and induced different effects on glycogen levels: glycogen increased in crabs fed with an HP diet, and decreased in crabs fed with an HC diet (Zanotelli et al., 2002). In other decapod species, 5-HT injection also increased hemolymph glucose levels of eyestalkless individuals (Bauchau and Mengeot, 1966; Lüschen et al., 1993; Sathyanandam et al., 2008). This effect can be explained by the stimulation of CHH secretion from other tissues outside the eyestalk (Chung et al., 1999; Chung and Zmora, 2008), but the direct effect of 5-HT on peripheral tissues cannot be excluded. In their classic paper, Bauchau et al. (1968) reported that 5-HT could increase glycogen phosphorylase activity in the Chinese crab *Eriocheir sinensis*. In this scenario, the following questions need to be addressed: Can 5-HT bind to receptors in the peripheral tissues of crustaceans, especially *N. granulata*? Does the metabolic pattern induced by the diet composition interfere with peripheral 5-HT effects?

Phylogenetic studies suggest that 5-HT receptors (5-HTRs) probably evolved, through gene duplication processes, around 750 million years ago. These evolutionary events occurred before the separation of vertebrates and invertebrates, which is estimated to have taken place around 600 million years ago, and predict that invertebrates and vertebrates may possess homologous 5-HT receptors (Tierney, 2001). However, this vast time span may have allowed differentiation of additional receptor subtypes within each class, which are assumed to have evolved independently in vertebrates and invertebrates (Tierney, 2001). In crustaceans, 5-HTRs with a pharmacological profile similar to mammalian 5-HT_1 and 5-HT_2 were described (Lee et al., 2000, 2001; Tierney and Mangiamele, 2001; Meeratana et al., 2006) and sequenced from the nervous system of the lobster *Panulirus interruptus* (5-$HT_{\alpha Pan}$ and 5-$HT_{2\beta Pan}$, Clark et al., 2004; Sosa et al., 2004), the crayfish *Procambarus clarkii* (5-$HT_{\alpha Pro}$ and 5-$HT_{2\beta Pro}$; Spitzer et al., 2008) and the freshwater prawn *Macrobrachium rosenbergii* (5-HT_{1Mac} and 5-HT_{2Mac}; Vázquez-Acevedo et al., 2009), and from the ovaries of the black tiger prawn *Penaeus japonicus* (5-HT_{1Pem}, Ongvarrasopone et al., 2006). In addition to the ovaries, the 5-HT_{1Pem} gene is expressed in the nervous system and in the heart, gills, stomach, muscle, and lymphoid tissues (Ongvarrasopone et al., 2006).

This study was designed to further investigate the hyperglycemic action of 5-HT in the crab *Neohelice granulata*. The effects of 5-HT injections on the levels of glucose in the hemolymph and of glycogen in the hepatopancreas, jaw muscle, and anterior and posterior gills of *N. granulata* crabs fed with HC or HP diet were measured. The effects of pre-treatment with mammalian 5-HT receptor antagonists (cyproheptadine and methiothepin) were also investigated. A series of *in vivo* experiments with ^{3}H-serotonin was carried out in order to investigate the presence of putative 5-HT receptors in peripheral tissues. Since gills were the tissue with the highest labeling in *in vivo* experiments, *in vitro* studies with isolated anterior and posterior gills were also conducted.

2. Materials and methods

2.1. Animals

N. granulata male crabs in stage C of the intermolt cycle, in accordance with the morphological criteria described by Drach and Tchemigovtzeff (1967), were collected in Lagoa de Tramandaí, a lagoon in the state of Rio Grande do Sul, Brazil (IBAMA license 39062–1). All the crabs used in this study were collected from late spring to early autumn. The crabs were kept in aquaria, with a natural photoperiod and a salinity of 20‰, at a temperature of 25 ± 2 °C, and under continuous aeration. After a week of acclimation in the laboratory, the animals were divided into two groups, one of which was fed with raw meat (a high-protein [HP] diet) while the other was fed with boiled rice (a carbohydrate-rich [HC] diet) daily at noon during two weeks.

2.2. Serotonin (5-HT) and antagonists in vivo bioassays

In order to investigate whether 5-HT can bind to different types of receptors, the mammalian 5-HT_1 antagonist, methiothepin, and 5-HT_2 antagonist, cyproheptadine, were used.

The first hemolymph sample (basal glucose) (100 μL) was collected from the base of the chelipeds at 10:00 a.m. (0.02 mL 10% potassium oxalate was used as an anti-clotting agent), and each crab was transferred to a numbered individual cage (10 × 10 × 10 cm^3) inside the aquarium. Following this procedure, the crabs remained undisturbed for about 3 h to reduce the stress caused by handling.

At the end of this period, both the crabs fed with the HC diet and those fed with the HP diet were sub-divided into four groups: (1) control group: each crab was injected twice with 100 μL saline with a 30-min interval between injections; (2) serotonin group: each crab was injected with 100 μL saline, followed by 100 μL 5-HT after 30 min; (3) methiothepin group: the crabs received 100 μL methiothepin, followed by another injection of 100 μL 5-HT after 30 min; and (4) cyproheptadine group: the crabs received 100 μL

cyproheptadine, followed by an injection of 100 μL 5-HT after 30 min. One and two hours after the second injection, the crabs were chilled, photographed to register postural alterations, and hemolymph and tissue samples were collected. The tissues (the hepatopancreas, the anterior and posterior gills, and the jaw muscle) were dissected on ice and frozen (−20 °C) in order to determine glycogen concentration.

Serotonin hydrochloride (Sigma H9523) 5.69×10^{-6} mol/crab (Vinagre, 1999) and the antagonists methiothepin mesylate salt (Sigma M149) 2.21×10^{-4}mol/crab) and cyproheptadine hydrochloride (Sigma C6022) 10^{-4}mol/crab were dissolved in crustacean physiological saline (NaCl 300 mM, KCl 10 mM, $MgCl_2$10mM, $CaCl_2$ 25 mM, H_3BO_3 8.8 mM and Tris buffer 0.1 M; 750 mOsm/L, pH 7.8). All the solutions were freshly prepared before the experiments to avoid oxidation.

2.3. ^{3}H-Serotonin (^{3}H-5-HT) and antagonists in vivo bioassays

In order to identify which tissues could have 5-HTRs, crabs fed with an HC or HP diet were sub-divided into three groups and received the following: (1) 100 μL of ^{3}H-5-HT (2×10^5 DPM [^{3}H]5-HT(5-hydroxy[G-3H]tryptamine creatinine sulfate) Perkin Elmer, freshly prepared in crustacean physiological saline; (2) 100 μL of ^{3}H-5-HT with 2.6×10^{-6} mol/crab of 5-HT (Zanotelli et al., 2002); and (3) 100 μL of ^{3}H-5-HT with 5.3×10^{-7} mol/crab of dopamine (Dopamine hydrochloride; Sigma H8502) (Zanotelli et al., 2002), respectively. Samples of hemolymph and tissues were collected exactly 30 min after each injection (Inohara, 2013). The crabs were chilled on ice for dissection, and samples of the hepatopancreas, heart, jaw muscle, and anterior and posterior gills were collected and prepared as described by Kucharski et al. (1997). The uptake of radioactivity by the tissues was expressed as the tissue to hemolymph ratio (T/H) (DPM/g of tissue/100 μL of hemolymph).

To determine the pharmacological characteristics of the putative binding sites, crabs fed with an HC or HP diet were sub-divided in three groups receiving the following: (1) 100 μL of ^{3}H-5-HT; (2) 100 μL of ^{3}H-5-HT with 2.21×10^{-4}mol/crab of methiothepin; and (3)100 μL of ^{3}H-5-HT with cyproheptadine10^{-4} mol/crab. Tissues were collected exactly 30 min after each injection, and procedures were the same as for specific binding.

2.4. ^{3}H-5-HT and antagonists in vitro bioassays

To evaluate the specificity of the binding sites, competition curves with increasing doses of 5-HT or antagonists were conducted. The crabs fed with either HC and HP diet (as indicated above) were chilled on ice for dissection, and samples of the anterior and posterior gills were collected. The gills incubation media were aerated with O_2:CO_2 (95:5 v/v) for 20 s and incubated for 20 min in 1.0 mL saline. After this period, the samples were dried in filter paper and transferred to incubation vials with 1.0 mL or 0.9 mL of 2.5 μCi ^{3}H-5-HT, freshly prepared in crustacean physiological saline; 0.1 mL of 5-HT (10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M) or cyproheptadine (10^{-4} M, 10^{-5} M, 10^{-6} M, and 10^{-7} M) or methiothepin (10^{-3} M, 10^{-4} M, 10^{-5}, and 10^{-6} M). The tubes were aerated with O_2:CO_2 (95:5 v/v) for 30 s and incubated at 25 °C for 30 min (time course study is shown in Supplement Fig. 3). After incubation, the gills were washed twice in saline, dried in filter paper, and transferred to vials containing scintillation liquid. Radioactivity was measured, and the results were first expressed as DPM/g of tissue and then converted to specific binding (SB): SB = TB − NSB, where TB refers to total binding (saline with ^{3}H-5-HT) and NSB refers to non-specific binding (experimental group). The results were expressed as ^{3}H-5-HT-specific binding (DPM/g tissue).

2.5. Biochemical analyses

Levels of glucose in the hemolymph samples were determined by the glucose-oxidase method (Labtest Kit), and results were expressed as mmol/L. Glycogen was extracted in accordance with Van Handel (1965), and values were expressed as glucose equivalents (the glucose-oxidase method), after acid hydrolysis, and neutralization with Na_2CO_4, in accordance with the Geary technique (Geary et al., 1981). Results were expressed as mg of glucose/g of tissue.

2.6. Statistical analyses

Results were expressed as the mean ± standard error of the mean (SEM). Student's *t* test or analysis of variance (ANOVA) in one, two, or three ways, in line with the aims of each experiment, was performed using the software PASW Statistics version 18.0, compatible with Windows 7. Before applying the ANOVA test, results were checked for normality (Kolmogorov–Smirnov) and homogeneity (Levene test) of data. ANOVA was followed by Bonferroni post-test. In the absence of homogeneity, data were mathematically transformed prior to the ANOVA test and were considered significant when $P < 0.05$. The competition curves of serotonin, methiothepin, and cyproheptadine in the *in vitro* experiments were analyzed by means of the Spearman correlation test.

3. Results

3.1. Serotonin and antagonists in vivo bioassays

The administration of 5-HT and cyproheptadine produced a typical 5-HT-like posture: contraction of the flexor muscles, resulting in an elevated posture with limbs and abdomen converging (Fig. 1A). Methiothepin injections caused the opposite effect, i.e., muscular relaxation. The administration of 5-HT 30 min after methiothepin reversed this effect, resulting in an intermediate 5-HT-like posture (Supplementary Fig. 1D).

The hemolymph glucose levels of the crabs are displayed in Fig. 1B and C. The three-way ANOVA analysis revealed differences between diets and times ($P < 0.05$). In HP diet crabs (Fig. 1B), 5-HT and methiothepin increased ($P < 0.05$, two-way ANOVA) glucose hemolymph levels, but cyproheptadine did not alter glucose levels ($P > 0.05$, two-way ANOVA). In crabs fed with an HC diet, all treatments increased glucose levels in the hemolymph (Fig. 1C, $P < 0.05$, two-way ANOVA).

Glycogen concentration in the hepatopancreas and jaw muscle of HP and HC crabs treated with 5-HT, methiothepin, and cyproheptadine is displayed in Table 1. The three-way ANOVA analysis revealed differences between the diets ($P < 0.05$), but there were no differences between times ($P > 0.05$) in both tissues. In the hepatopancreas of HP crabs, a two-way ANOVA analysis demonstrated that the 5-HT and cyproheptadine groups have higher glycogen levels than both the control and methiothepin groups. In the anterior gills of HP crabs (Table 2), reduced glycogen values ($P < 0.05$) in relation to control group at 120 min are evident in methiothepin treated group. No significant ($P > 0.05$) variation in glycogen levels was observed in the anterior and posterior gills of HC crabs.

3.2. ^{3}H-5-HT and antagonists in vivo bioassays

The uptake of radioactivity was highest in the posterior and anterior gills, followed by the heart, jaw muscle, and hepatopancreas, which displayed the lowest levels of radioactivity. The results of crabs fed with HP diet (Fig. 2A) were different from the crabs fed with HC diet (Fig. 2B) (three-way ANOVA, $P < 0.05$). In the anterior and posterior gills of HP crabs (Fig. 2A), 5-HT inhibited ^{3}H-5-HT binding ($P < 0.05$, two-way ANOVA, Bonferroni Test). In the anterior gills, dopamine also inhibited ^{3}H-5-HT binding. In the jaw muscle, 5-HT inhibited ^{3}H-5-HT binding ($P < 0.05$) in relation to the dopamine-treated group ($P < 0.05$, two-way ANOVA, Bonferroni Test). In the heart, no significant differences in ^{3}H-5-HT binding were recorded ($P > 0.05$). Dopamine increased ^{3}H-5-HT binding ($P < 0.05$) in the hepatopancreas. No significant differences were registered in the crabs fed with an HC diet ($P > 0.05$) (Fig. 2B).Since there were differences in ^{3}H-5-HT binding which were

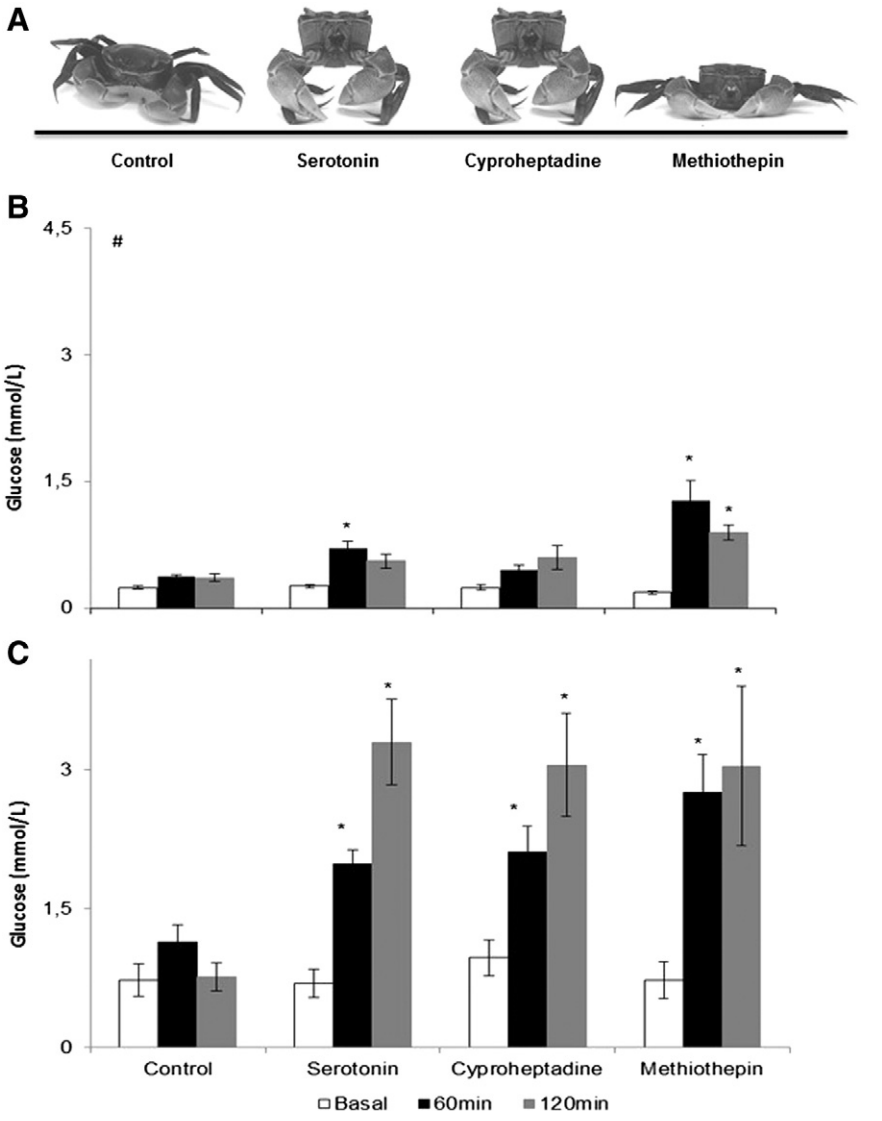

Fig. 1. Effects of injection of serotonin and antagonists on the posture and hemolymph glucose of *Neohelice granulata*. Each crab received 2 injections according to the specific treatment: Control (saline/saline); Serotonin (saline/serotonin); Cyproheptadine (cyproheptadine/serotonin); Methiothepin (methiothepin/serotonin). (A) Postural alterations 120 min after injections. (B) Hemolymph glucose levels in crabs fed a high-protein (HP) diet at baseline and after 60 or 120 min of second injection: Control ($n = 38/19/14$); Serotonin ($n = 42/20/19$); Cyproheptadine ($n = 31/15/12$); Methiothepin ($n = 16/7/8$). (C) Hemolymph glucose levels in crabs fed a high-carbohydrate (HC) diet at baseline and after 60 or 120 min of second injection. Control ($n = 33/18/15$); Serotonin ($n = 36/18/18$); Cyproheptadine ($n = 27/13/13$); Methiothepin ($n = 15/7/7$). Values represent mean ± SEM. #Significant difference between diets ($P < 0.05$, three-way ANOVA). *Significant difference in relation to control group ($P < 0.05$, two-way ANOVA, followed by Bonferroni pos hoc test).

caused by diet composition, a binding assay was carried out on a group of field crabs on the day following their arrival from the field (Supplementary Fig. 2). The methods of this experiment were the same described for the crabs fed with HC or HP diet, with the exception that this group of crabs were not fed (24 h fasting). Treatment with 5-HT reduced ^{3}H-5-HT binding ($P < 0.05$, two-way ANOVA, Bonferroni test) in the jaw muscle, heart, and hepatopancreas. However, no significant differences were registered in the gills ($P > 0.05$).

Methiothepin/^{3}H-5-HT treatment increased ($P < 0.05$, two-way ANOVA, Bonferroni test) binding of ^{3}H-5-HT in the anterior and

Table 1
Effects of injection of serotonin and antagonists on glycogen concentration (mg/g) of hepatopancreas and jaw muscle of *Neohelice granulata* previously fed a high-carbohydrate (HC) or high-protein (HP) diet, 60 and 120 min after second injection.

Group 1st injection/2nd injection	Hepatopancreas HC		Hepatopancreas HP #		Jaw muscle HC		Jaw muscle HP #	
	60	120	60	120	60	120	60	120
Control, Saline/saline	10.3 ± 1.2 (18)	11.2 ± 1.4 (15)	1.8 ± 0.4 (19)	1.6 ± 0.3 (16)	16.7 ± 1.5 (17)	18.1 ± 2.3 (14)	5.9 ± 1.0 (18)	5.2 ± 0.8 (16)
5-HT, Saline/5-HT	12.8 ± 1.0 (17)	13.2 ± 0.7 (15)	3.2 ± 0.4* (20)	3.2 ± 0.5* (18)	19.3 ± 2.5 (18)	17.5 ± 1.3 (15)	4.2 ± 0.6 (20)	4.7 ± 0.5 (18)
Cyproheptadine, Cyproheptadine/5-HT	13.8 ± 1.0 (13)	12.2 ± 0.9 (12)	2.4 ± 0.4* (14)	3.4 ± 0.4* (12)	17.3 ± 1.6 (12)	19.2 ± 4.5 (13)	3.8 ± 0.5 (15)	6.0 ± 2.2 (11)
Methiothepin, Methiotepin/5-HT	10.8 ± 1.4 (8)	9.8 ± 1.4 (8)	2.7 ± 0.9 (7)	1.8 ± 0.4 (9)	22.2 ± 2.3 (8)	16.9 ± 2.4 (8)	5.0 ± 0.5 (7)	3.2 ± 0.5 (9)

#Significant difference between diets ($P < 0.05$; three-way ANOVA).
*Significant difference between in relation to control group ($P < 0.05$, two-way ANOVA followed by Bonferroni post-test).

Table 2
Effects of injection of serotonin and antagonists on glycogen concentration (mg/g) in anterior and posterior gills of *Neohelice granulata* previously fed a high-carbohydrate (HC) or high-protein (HP) diet, 60 and 120 min after second injection.

Group 1st injection/2nd injection	Anterior gills HC		Anterior gills HP		Posterior gills HC		Posterior gills HP	
	60	120	60	120	60	120	60	120
Control, saline/saline	5.4 ± 0.9 (4)	5.3 ± 2.0 (4)	7.7 ± 1.3 (4)	6.2 ± 0.5[a] (4)	9.5 ± 1.3 (4)	8.8 ± 2.0 (4)	12.7 ± 1.1 (4)	10.4 ± 0.6 (4)
5-HT, saline/5-HT	4.6 ± 0.7 (6)	3.3 ± 0.9 (7)	6.1 ± 1.0 (6)	6.3 ± 0.9[a] (7)	12.9 ± 2.0 (6)	8.7 ± 2.1 (7)	9.8 ± 1.5 (6)	9.8 ± 1.4 (7)
Cyproheptadine, cyproheptadine/5-HT	5.7 ± 0.6 (6)	5.8 ± 1.2 (6)	7.4 ± 1.0 (6)	5.9 ± 1.5[a] (6)	12.7 ± 1.6 (6)	12.1 ± 1.8 (6)	12.0 ± 1.4 (6)	9.1 ± 2.5 (5)
Methiothepin, methiotepin/5-HT	4.7 ± 1.1 (6)	5.5 ± 1.3 (6)	5.3 ± 0.5 (6)	2.9 ± 1.0[b] (6)	11.0 ± 1.8 (6)	13.0 ± 1.1 (6)	8.2 ± 0.6 (6)	5.9 ± 1.7 (6)

Letters: denote significant differences between treatments ($P < 0.05$, two-way ANOVA followed by Bonferroni post-test).

posterior gills of HP diet crabs (Fig. 3A) in relation to treatment with ^{3}H-5-HT alone. Cyproheptadine treatment reduced ($P < 0.05$) ^{3}H-5-HT binding in the hepatopancreas and posterior gills in relation to the methiothepin group. In the jaw muscle and heart of HP crabs, no significant differences were registered ($P > 0.05$). The results of HC crabs were different (three-way ANOVA, $P < 0.05$) from those of HP crabs (Fig. 3B). In the jaw muscle of HC crabs, methiothepin reduced ^{3}H-5-HT binding (two-way ANOVA, $P < 0.05$), whilst in the other tissues no significant differences were found ($P > 0.05$).

3.3. ^{3}H-5-HT and antagonists in vitro bioassays

In the crabs fed with the HP diet (Fig. 4), increasing doses of 5-HT decreased ^{3}H-5-HT binding in a dose-dependent way both in anterior ($r_{s(0.05;4)} = -0{,}416$) and posterior ($r_{s(0.01;4)} = -0{,}614$) gills. In the HC group, 5-HT strongly inhibited ^{3}H-5-HT-specific binding in relation to total binding. The average inhibition was by 85% and 95% in the anterior and posterior gills, respectively. However, this inhibition was not dose dependent.

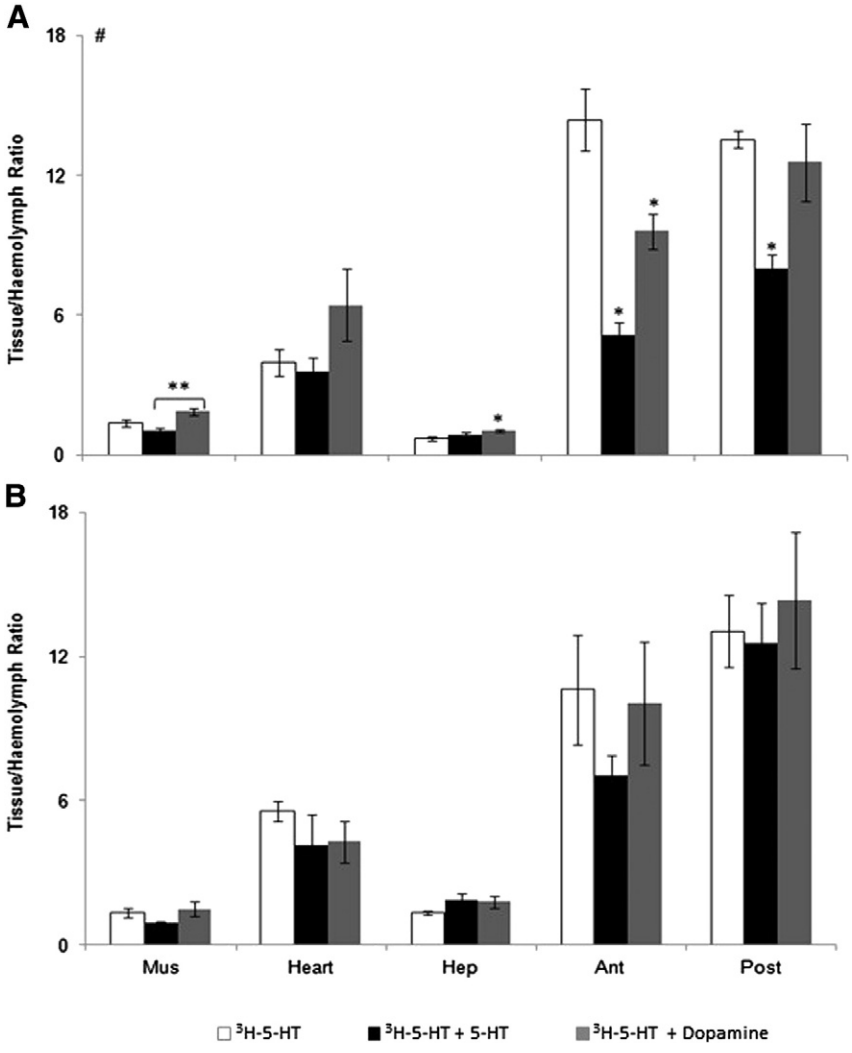

Fig. 2. Binding of ^{3}H-5-HT (tissue/hemolymph ratio: DPM/g tissue/100 μL hemolymph) in peripheral target tissues of the *Neohelice granulata* crab: jaw muscle (Mus), heart, hepatopancreas (Hep), anterior gills (Ant), and posterior gills (Post). (A) Crabs fed with high-protein (HP) diet. (B) Crabs fed with carbohydrate-rich (HC) diet. Treatment groups: White columns:^{3}H-5-HT (crabs treated with ^{3}H-5-HT (200,000 DPM/100 μL saline) ($n = 5$–7); Black columns: crabs treated with ^{3}H-5-HT (200,000 DPM/100 μL saline) and serotonin (5.69×10^{-3} mol/crab) ($n = 5$–7); Gray columns: crabs treated with ^{3}H-5-HT (200,000 DPM/100 μL saline) and dopamine (5.3×10^{-3} mol/crab) ($n = 5$). Tissue samples collected 30 min after injections. Columns and bars represent means ± SEM. #Significant difference between diets ($P < 0.05$, three-way ANOVA). *Significant difference compared to H^3-5-HT group. **Significant difference in relation to dopamine group.

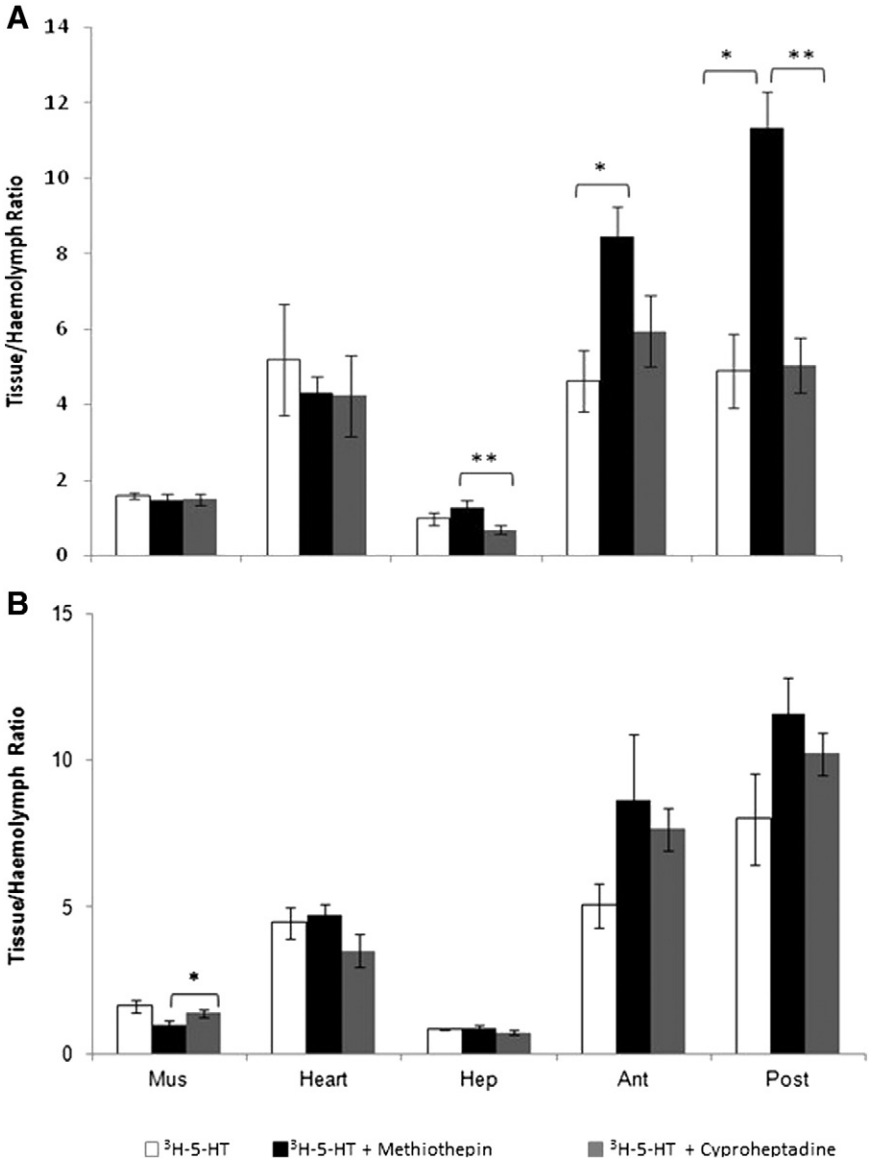

Fig. 3. Effects of antagonists on the binding of ^{3}H-5-HT (tissue/hemolymph ratio: DPM/g tissue/100 μL hemolymph) in peripheral target tissues: jaw muscle (Mus), heart, hepatopancreas (Hep), anterior gills (Ant), and posterior gills (Post) of *Neohelice granulata* crabs fed with high-protein (A) or high-carbohydrate (B) diets. White columns: crabs treated with ^{3}H-5-HT (200,000 dpm/100 μL saline) ($n = 4$); Black columns: crabs treated with ^{3}H-5-HT (200,000 dpm/100 μL saline) and methiothepin (2.21×10^{-2} mol/crab) ($n = 8$) Gray columns: crabs treated with ^{3}H-5-HT (200,000 DPM/100 μL saline) and cyproheptadine (10^{-3} mol/crab) ($n = 8$). Columns and bars represent means ± SEM. #Significant difference between diets ($P < 0.05$, three-way ANOVA). *Significant difference compared to group ^{3}H-5-HT. **Significant difference in relation to methiothepin group.

In the anterior gills of the crabs fed with the HC diet, increasing doses of methiothepin (Supplementary Fig. 4A) decreased ^{3}H-5-HT-specific binding in a dose-dependent manner ($r_{s(0.05;4)} = -0{,}629$); however, this inhibition was not dose dependent in the crabs fed with the HP diet. In the posterior gills of both groups of diets (Supplementary Fig. 4B), binding inhibition by methiothepin was also independent of methiothepin concentration.

Increasing doses of cyproheptadine inhibited ^{3}H-5-HT-specific binding in both types of gills. However, this inhibition was not dose dependent (Supplementary Fig. 4C and D).

In order to illustrate the inhibition of ^{3}H-5-HT binding by the antagonists in a simplified figure, a single dose of each antagonist was selected to build Fig. 5.These results were analyzed with Student's *t* test. In anterior gills, the presence of 10^{-6} M methiothepin inhibited ^{3}H-5-HT binding by 56% in the crabs fed with the HP diet (Fig. 5A, $P < 0.01$), and by 97% in the crabs fed with HC diet ($P < 0.05$). The presence of methiothepin in posterior gills inhibited binding by 76% and 90% in the group fed with HP and HC diet, respectively. In the anterior gills, the presence of 10^{-7} M cyproheptadine inhibited the average ^{3}H-5-HT-specific binding by 70% ($P < 0.05$) in the crabs fed with the HC diet and by 50% ($P < 0.01$) in the crabs fed with the HP diet (Fig. 5B). In posterior gills, cyproheptadine inhibited the average ^{3}H-5-HT-specific binding by 65% ($P < 0.05$) in the crabs fed with the HC diet and by 80% ($P < 0.01$) in the crabs fed with the HP diet.

4. Discussion

N. granulata lives in a highly variable environment with oscillations in temperature, salinity, and food availability (Spivak, 2010). This environment probably contributed to the evolution of a complex mechanism for the maintenance of glucose homeostasis. This study suggests that serotonin, in addition to its well-known effect on CHH secretion, can also influence glucose homeostasis through binding with at least two types of 5-HTRs in the peripheral tissues of *N. granulata* and that the binding of 5-HT to these receptors is affected by the composition and frequency of the diet fed to the crabs.

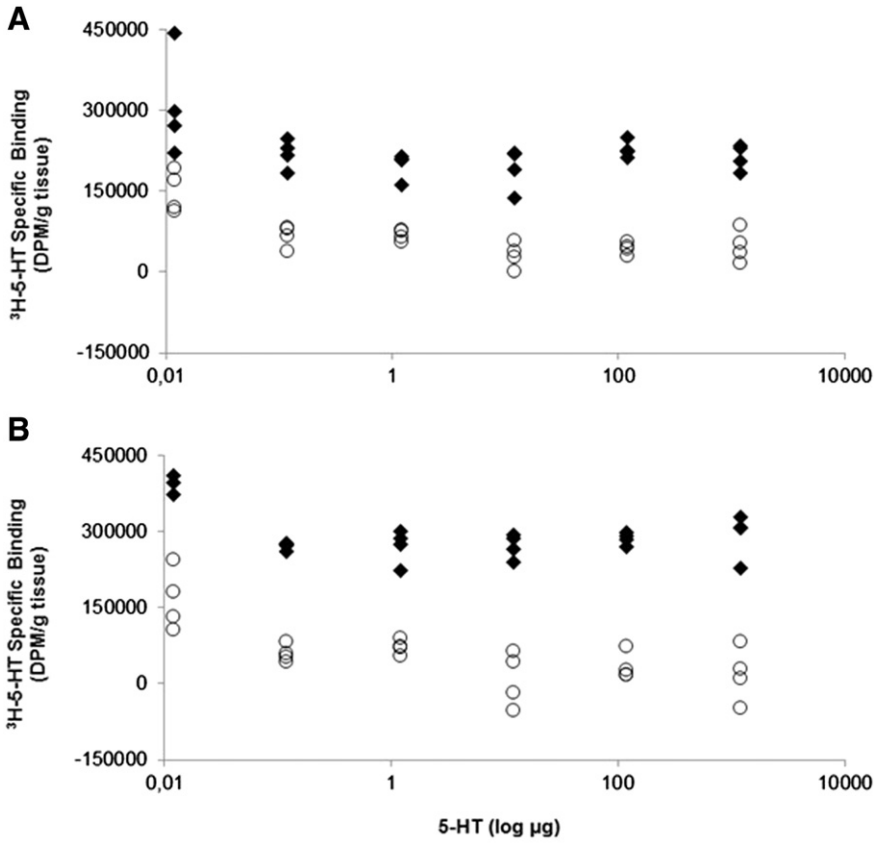

Fig. 4. Binding competition curves between ^{3}H-5-HT and 5-HT in anterior (A) and posterior (B) gills of *Neohelice granulata* crabs fed an HP (♦) or HC (○) diet. Treatments: saline with ^{3}H-5-HT ($n = 4/4$); saline with ^{3}H-5-HT and 5-HT in increasing doses: 10^{-7} M ($n = 4/4$), 10^{-6} M ($n = 4/4$), 10^{-5} M ($n = 4/4$), 10^{-4} M ($n = 4/4$), and 10^{-3} M ($n = 4/4$). The data were analyzed using the Spearman correlation test. In the crabs fed with the HC diet, the correlation was not significant, but in the crabs fed with the HP diet, it was significant both in the anterior ($r_{s(0.05;4)} = -0{,}416$) and posterior ($r_{s(0.01;4)} = -0{,}614$) gills.

As already outlined in previous papers (Kucharski and Da Silva, 1991a, 1991b; Vinagre and Da Silva, 1992), hemolymph glucose levels are higher in *N. granulata* crabs fed with an HC diet than in those fed with an HP diet. As expected, the administration of 5-HT increased glucose levels in the hemolymph (Vinagre, 1999; Santos et al., 2001; Zanotelli et al., 2002), demonstrating that 5-HT influences glucose homeostasis in *N. granulata*. There are three possible hypotheses for explaining the effect of 5-HT: (1) it may be an indirect effect, since 5-HT stimulates the secretion of CHH (crustacean hyperglycemic hormone) from the eyestalk; (2) it may be the direct effect of 5-HT through the activation of 5-HTRs on peripheral tissues; (3) it may be both hypotheses operating in association. In order to investigate whether 5-HT can interact with more than one type of receptors on peripheral tissues, two widely recognized mammalian 5-HT receptor antagonists were chosen: cyproheptadine, which interacts with the 5-HT$_2$ receptor family, and methiothepin which binds with the 5-HT$_1$ receptor family. Methiothepin did not modify the 5-HT hyperglycemic effect in animals fed with both diets, but when cyproheptadine was injected prior to 5-HT, crabs fed with the HP diet did not display significant increases in hemolymph glucose. A similar effect of cyproheptadine was noted in the shrimp *Squilla mantis* and in the crayfish *Astacus leptodactylus*, which were fed with high-protein diets, i.e., shrimp and fish, and beef and liver, respectively (Lorenzon et al., 2004). In the crayfish *P. clarkii*, the injection of DOI (1-[2, 5 -dimetóxi-4-iodofenil]isopropilamina), a 5HT$_{2A}$ receptor agonist, caused hyperglycemia, while the 5HT$_{2A}$ receptor antagonist ketanserin blocked 5-HT-induced hyperglycemia (Lee et al., 2000). It may be that the hyperglycemic effect of 5-HT on *N. granulata* is influenced by the 5-HT interaction with a putative receptor which has pharmacological characteristics similar to those of the mammalian 5-HT$_2$ family, and this binding is influenced by the composition of the diet administered to the crabs.

The levels of glycogen in the hepatopancreas and jaw muscle are higher in *N. granulata* crabs fed with an HC diet than in crabs fed with an HP diet (Kucharski and Da Silva, 1991a, 1991b; Vinagre and Da Silva, 1992). These diets did not influence the glycogen levels of the anterior and posterior gills in this paper, as was already noted by Vinagre and Da Silva (1992). The levels of glycogen in the tissues of HC crabs were not affected by 5-HT, cyproheptadine, or methiothepin treatments. As noted by Zanotelli et al. (2002), 5-HT increased glycogen levels in the hepatopancreas of HP crabs, and the same effect occurred with cyproheptadine. Treatment with methiothepin followed by 5-HT altered glycogen levels in relation to the 5-HT crabs, suggesting that methiothepin may have blocked the 5-HT effect. The only tissue where glycogen levels were reduced in relation to control were the anterior gills of HP- fed crabs 2 h after the treatment with methiothepin and 5-HT. These results suggest the presence of 5-HT$_1$-like receptors in the hepatopancreas and gills. However, the hyperglycemic action of 5-HT cannot be explained by glycogenolysis, as is to be expected from the results of Bauchau et al. (1968), which noted that 5-HT increased glycogen phosphorylase activity in the Chinese crab *E. sinensis*. Other metabolic pathways, such as gluconeogenesis or a reduction in glucose utilization by peripheral tissues, may be responsible for the hyperglycemic effect.

In mammals, Watanabe et al. (2010) reported hyperglycemia following 5-HT injections in mice, accompanied by increases in glycogen concentration and PEPCK (phosphoenolpyruvate carboxykinase—the main gluconeogenic enzyme) activity in the liver. This increase in PEPCK activity was also reported in pregnant rats fed with the 5-HT

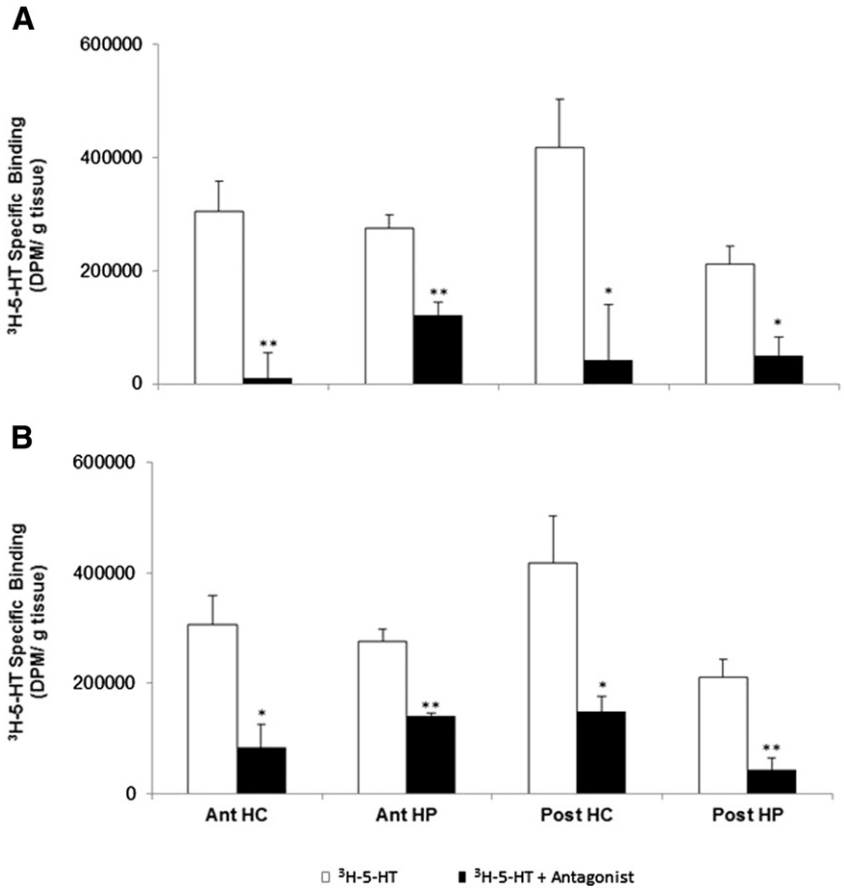

Fig. 5. The effect of 10^{-6} M methiothepin (A) and 10^{-7} M cyproheptadine (B) on H^3-5-HT-specific binding in the anterior (Ant) and posterior (Post) gills of *Neohelice granulata* crabs fed with high-protein (HP) or carbohydrate-rich (HC) diet. White columns: ^{3}H-5-HT (crabs treated with ^{3}H-5-HT (200,000 DPM/100 μL saline); black columns: crabs treated with ^{3}H-5-HT (200,000 DPM/100 μL saline) and antagonist. Values represent mean ± SEM ($n = 3$–4). *Significant difference between treatments at $P < 0.05$ level (Student's t test). **Significant difference between treatments at $P < 0.01$ level (Student's t test).

precursors, tryptophan or hydroxitryptophan (Laporta et al., 2013). The latter also described increases in the levels of 5-HT in plasma, the liver, and the mammary gland. In the *N. granulata* crab, high gluconeogenic capacity and PEPCK activities were reported in the hepatopancreas, jaw muscle, and anterior and posterior gills (Oliveira and Da Silva, 1997; Vinagre and Da Silva, 2002; Schein et al., 2004). The presence of gluconeogenesis in *N. granulata* was first noted in the hepatopancreas (Oliveira and Da Silva, 1997), when glucose synthesis was reported using ^{14}C-alanine and ^{14}C-lactate as substrates. It was suggested that this high gluconeogenic activity allows *N. granulata* to promptly react to extreme alterations in environmental factors, such as modifications in temperature, salinity, tidal cycles, and food resources by producing adequate glucose, which is the crabs' main energetic substrate. This hypothesis is also supported by the fact that, in the hepatopancreas of *N. granulata*, 5-HT injections decreased free glucose levels in intact crabs fed with either an HC or HP diet (Zanotelli et al., 2002). This reduction in free glucose, associated with the increase in glycogen concentration, can be explained by gluconeogenesis followed by glycogen synthesis, i.e., the process of glyconeogenesis. In the muscles of *N. granulata*, it has been demonstrated that glyconeogenesis is also involved in the reduction of circulating lactate levels during refeeding after a starvation period (Pellegrino et al., 2008).

In this study, the injection of 5-HT caused a typical 5-HT-like posture both in HC and HP crabs (Fingerman and Nagabhushanam, 1992; Tierney and Mangiamele, 2001; Vinagre et al., 2004; Wu and Cooper, 2012). The administration of methiothepin resulted in the opposite posture, which was characterized by muscular relaxation. The administration of 5-HT after methiothepin resulted in an intermediate posture, suggesting a partial blockage of the 5-HT effect in the muscles. This result also suggests the presence of 5-HT_1-like receptors in the peripheral nervous system, and/or appendix muscles of *N. granulata*. In the *P. clarki* crayfish, an immunocytochemical study with anti-5-HT_{1Crus} revealed the presence of this receptor throughout the peripheral nervous system and abdominal flexor muscle, suggesting its participation in postural control (Spitzer et al., 2005).The administration of cyproheptadine did not alter 5-HT-like posture in this study. In the *N. granulata* crab, cyproheptadine reduced the escape reaction 30 min and 24 h after its administration (Aggio et al., 1996). However, the long-term sensitization induced by 5-HT was not blocked by cyproheptadine, suggesting the existence of two distinct 5-HTRs in the nervous system of *N. granulata*.

Binding of ^{3}H-5-HT *in vivo* was carried out in order to identify the location of peripheral 5-HTRs. The administration of 5-HT in combination with ^{3}H-5-HT decreased binding significantly in the jaw muscle, hepatopancreas, and gills of HP diet-fed crabs, thus demonstrating the specificity of the binding in these tissues. In the HC crabs, 5-HT did not affect ^{3}H-5-HT binding in any of the tissues. In the hepatopancreas, heart and jaw muscle samples from crabs freshly collected from the field, the administration of 5-HT in combination with ^{3}H-5-HT decreased binding significantly, thus also demonstrating the specificity of the binding in these tissues. Overall, these results suggest the presence of 5-HTRs in peripheral tissues of *N. granulata*. In addition, nutritional or biochemical factors associated with the diets administered influenced ^{3}H-5-HT binding. In the jaw muscle of HC crabs, the reduction of ^{3}H-5-HT binding

caused by methiothepin together with the methiothepin postural effect suggests the presence of 5-HT_1-like receptors in the muscular tissues of *N. granulata*. As regards the heart, cyproheptadine and methiothepin did not influence ^{3}H-5-HT binding, suggesting the presence of a distinct type of 5-HTR.

Binding of ^{3}H-5-HT was higher in both types of gills in relation to other tissues, with the posterior gills displaying the highest levels of radioactivity. A similar result was obtained with I^{125}-Insulin binding in the gills of *N. granulata* (Kucharski et al., 1997). In the Chinese crab *Eriocher sinensis*, the posterior gills also displayed higher levels of ^{3}H-SCH233903 (a D_1 receptor antagonist) binding than the anterior gills (Mo et al., 2002). Serotonin and dopamine are considered to be important regulators of the osmoregulatory processes in the gills of decapods (Mo et al., 2002; Halperin et al., 2004; Liu et al., 2008). In the anterior gills of the HP crabs, the administration of dopamine in combination with ^{3}H-5-HT decreased binding. The presence of D_1-*like* and D_2-*like* receptors in the posterior gills of *N. granulata* was previously proposed by Halperin et al. (2004). Dopamine receptors in gills were also reported in *Birgus latro* (Morris, 2001) and *E. sinensis* (Mo et al., 2002) crabs. Dopamine modulates ion transport in the posterior gills of *N. granulata* by means of a highly complex mechanism, which involves a stimulatory phase mediated by cAMP-PKA and a rapid deactivation phase which brings Na^+/K^+-ATPase to resting values. Both of these regulatory phases may be explained by the interaction of receptors linked to different G proteins: D_1-like receptors are probably linked to Gs protein, while D_2-like receptors are probably linked to Gi protein (Genovese et al., 2006). The fact that dopamine reduced ^{3}H-5-HT binding suggests that this monoamine also has affinity with 5-HTRs, or that dopamine, in binding to its own receptors, can have a modulatory action on 5-HTRs. In this study, dopamine also influenced ^{3}H-5-HT binding in the hepatopancreas of HP crabs, lending support to the hypothesis of this modulatory action.

In the studies *in vivo*, methiothepin increased ^{3}H-5-HT binding in both anterior and posterior gills of HP-fed crabs, while cyproheptadine had no related influence. In mammals, methiothepin is considered to be an antagonist of all types of receptors of the 5-HT_1 family. Receptor 5-HT_{1B} can act as an auto-receptor, inhibiting 5-HT secretion (Newman-Tancredi et al., 2003; Pytliak et al., 2011). Binding of ^{3}H-5-HT in *in vivo* experiments may be influenced by alterations in endogen 5-HT levels. Therefore, if 5-HT_1-like receptors in the gills of *N. granulata* have pharmacological characteristics similar to mammal 5-HT_{1B}, the inhibition of endogen 5-HT secretion may explain the increase in ^{3}H-5-HT binding with methiothepin treatment. In crustaceans, 5-HT reuptake seems to be involved in the control of behavior, since some 5-HT behavioral effects can be altered by fluoxetine, an inhibitor of 5-HT reuptake (Huber, 2005; Vázquez-Acevedo et al., 2009). In *Carcinus maenas*, fluoxetine caused a different effect from 5-HT on locomotor behavior (Mesquita et al., 2011). In this case, the increase in circulating endogen 5-HT levels caused by fluoxetine may have activated distinct 5-HTRs from the injection of exogenous 5-HT, thereby explaining the difference between the two effects.

In both types of gills of crabs fed with either diet, 5-HT inhibited ^{3}H-5-HT-specific binding, thus demonstrating the specificity of the binding assay. The inhibition was dose dependent only in the crabs fed with the HP diet, suggesting that even under *in vitro* conditions; diet affects ^{3}H-5-HT binding. This constitutes the first evidence of 5-HTRs in the gills of *N. granulata*, suggesting that 5-HT, as well as dopamine, may have an important role in the neuroendocrine control of osmoregulatory and metabolical processes in the gills of this crab.

Methiothepin inhibition of ^{3}H-5-HT-specific binding *in vitro* in both the anterior and posterior gills suggests the presence of 5-HT_1-like receptors in *N. granulata*. In crustaceans, 5-HT_1-like receptors were cloned and sequenced in *P. clarkii* (Spitzer et al., 2008), *M. rosenbergii* (Vázquez-Acevedo et al., 2009), *P. interruptus* (Sosa et al., 2004), and *Penaeus monodon* (Ongvarrasopone et al., 2006). Crustacean 5-HT_1 receptors contain key structural elements typical of the 5-HT_1 receptor superfamily, which are preserved in arthropods (Spitzer et al., 2008). In *P. monodon*, the 5-HT_{1Pem} gene is expressed in all the tissues studied, and in the ovaries, it is constitutively expressed during maturation. The transient expression of 5-HT_{1Pem} in HEK293 cells demonstrated a saturable ^{3}H-5-HT binding (Ongvarrasopone et al., 2006).

Cyproheptadine can also act as an antagonist on both types of gills, suggesting the existence of 5-HT_2-like receptors in the gills of *N. granulata*. Crustacean 5-HT_2-like receptors were identified in *P. clarkii* (Spitzer et al., 2008), in the freshwater prawn *M. rosenbergii* (Vázquez-Acevedo et al., 2009) and in *P. interruptus* (Clark et al., 2004). In *P. interruptus*, 5-$HT_{2\beta Pan}$ is more homologous to 5-HT_2 type receptors than *P. clarkii* and exhibits a dose-dependent response to 5-HT. When expressed in human HEK cells, it signals via G_q and is constitutively active. This receptor is present in the synaptic neuropil of the stomatogastric ganglion and axon terminals to the stomatogastric neurons, and therefore is probably involved in modulating the motor output of stomatogastric networks (Clark et al., 2004).

Previous studies with *N. granulata* showed that the carbohydrate or protein contents of the diets previously administered to the crabs induce different metabolic adjustments during nutrient, oxygen, and osmotic stresses (Schein et al., 2004; Pellegrino et al., 2008; Marqueze et al., 2011). However, we still lack information about the neuroendocrine factors involved in these metabolic adjustments. The presence of 5-HTRs in the gills of *N. granulata* suggests that 5-HT may have a metabolic function, as well as a role in the osmoregulatory processes.

In conclusion, this study highlights the importance of serotonin in the control of glucose homeostasis in crustaceans and provides evidences of 5-HT binding sites in the heart, jaw muscle, hepatopancreas, and anterior and posterior gills of *N. granulata*, suggesting that, as well as its known stimulatory effect on CHH secretion, 5-HT can have direct metabolical effects in peripheral tissues. Two types of 5-HT receptors are present in the gills of *N. granulata*: a 5-HT_1-like receptor with pharmacological characteristics similar to mammalian 5-HT_1 receptors, and a 5-HT_2-like receptor with pharmacological characteristics similar to mammalian 5-HT_2 receptors, suggesting a role of 5-HT in the control of the osmoregulatory process. Diet composition and regularity of administration can influence 5-HT binding to these receptors. However, the mechanisms responsible for these effects remain to be determined. Further studies, such as binding assays with isolated gill membranes, description of the second messenger systems and metabolic functions as well as sequencing of these receptors, are necessary for a better understanding of these results.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2015.03.012.

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